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O-antigenic chains of lipopolysaccharide prevent binding of antibody molecules to an outer membrane pore protein in *Enterobacteriaceae*

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The accessibility of outer membrane pore protein PhoE to antibody molecules at the cell surface of intact cells of various *Enterobacteriaceae* was investigated. Significant antibody binding was observed for only two of the nine strains tested. Analysis of the lipopolysaccharide by sodium dodecyl sulphate gel electrophoresis revealed a clear correlation between the presence of an O-antigenic side chain and the inability to bind PhoE protein-specific antibodies. As mutants that lack the O-antigen chain appeared to have acquired the ability to bind antibody, it must be concluded that the presence of O-antigenic chains of lipopolysaccharide prevents binding of antibodies to PhoE protein at the surface of intact cells. The relevance of this conclusion for the potential use of enterobacterial outer membrane pore proteins as vaccine components is discussed.

Key words: Outer membrane proteins; lipopolysaccharide; bacterial vaccines; bacterial cell surface; monoclonal antibodies.

Introduction

The outer membrane of Gram-negative bacteria contains a number of proteins which form pores through which small hydrophilic solutes can pass.¹ PhoE pore protein of the *E. coli* K-12 outer membrane is synthesized when cells are grown under phosphate limitation.² Its amino acid sequence shows a high degree of homology with two other outer membrane pore proteins.^{3,4} As part of our study on the structure–function relationships of PhoE protein we have isolated monoclonal antibodies directed against the cell surface-exposed part of the protein.⁵ Five out of six isolated monoclonals were found to bind to intact cells of *E. coli* K-12 derivatives which express *phoE* genes derived from 13 strains of various *Enterobacteriaceae*.⁵ If this apparent conservation of the cell surface-exposed part is a general property of outer membrane proteins they are good candidates for vaccine components. In fact, the possibility of using outer membrane proteins as vaccine components is under investigation in several laboratories.^{6–11} However, as pore proteins are closely associated with lipopolysaccharide (LPS), the presence of the long O-antigenic chains of LPS could prevent access of antibody molecules to the pore proteins and thereby limit their usefulness as protective vaccine.

We have used monoclonal and polyclonal antibodies directed against PhoE pore protein of *E. coli* K-12 to investigate this question. The results show that in *Enterobacteriaceae* the presence of O-antigen prevents antibody binding to this outer membrane protein.

Results

No binding of PhoE protein-specific antibodies to whole cells with O-antigen carrying LPS

Whole cells of *E. coli* K-12 derivatives which express *phoE* genes derived from 13 strains of various *Enterobacteriaceae* can bind five out of six isolated anti-PhoE monoclonal antibodies.⁵ In order to investigate the role of the O-antigen moiety of the LPS molecule, the binding of PhoE protein-specific antibodies to *E. coli* K-12 cells, which lack the O-antigen, was compared with the binding to some of the original strains from which the cloned *phoE* genes had been obtained. Binding of antibodies to the cell surface of mutants of nine strains which produce PhoE protein constitutively was tested by the cell immuno radio assay (CIRA).⁵ Binding was found only in case of *E. coli* strain U4 and, to a lesser extent, *Klebsiella aerogenes* (Table 1). The presence of an O-antigen moiety in the LPS molecules of these strains was investigated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis of proteinase K-treated cell envelopes. Ladder structures typical for O-antigen-carrying LPS were found for seven of the nine strains tested (Fig. 1). Only *E. coli* strain U4 (Fig. 1, lane D) and *Klebsiella aerogenes* (Fig. 1, lane E) synthesize only short LPS like *E. coli* K-12 (Fig. 1, lane A). The ability of cells to bind antibodies was therefore found to be absolutely correlated with the absence of O-antigen (compare Table 1 and Fig. 1).

Table 1 Binding of monoclonal and polyclonal antibodies to PhoE protein-constitutive strains and their derivatives with reduced amounts of O-antigen (*)

Strain	Presence of O-antigen ^a	Binding of monoclonal antibody PPI-4 (counts per min)	Binding of polyclonal antibodies (counts per min)
<i>Escherichia coli</i> CE1321 ^b	—	52 300	72 400
<i>Escherichia coli</i> CE1323	—	1900	1400
<i>Escherichia coli</i> U4 (0134)	—	104 400	129 600
<i>Escherichia coli</i> U20 (015)	+	1500	1700
<i>Escherichia coli</i> U20-3*	±	28 100	163 400
<i>Escherichia coli</i> F2 (015)	+	2000	1400
<i>Escherichia coli</i> F2-1*	±	59 800	196 000
<i>Escherichia coli</i> F3 (041)	+	4500	4100
<i>Escherichia coli</i> F3-2*	—	73 200	210 900
<i>Escherichia coli</i> F8 (0156)	+	2400	1700
<i>Escherichia coli</i> F8-5*	—	12 500	41 800
<i>Escherichia coli</i> S4 (011)	+	3700	n.t. ^c
<i>Klebsiella pneumoniae</i>	+	1200	n.t.
<i>Klebsiella aerogenes</i>	—	11 800	34 900
<i>Citrobacter freundii</i>	+	1500	2000
<i>Citrobacter freundii</i> -2*	—	23 600	109 000

^a The amount of O-antigen was apparently normal (+), significantly reduced (±) or not detected (—), see Fig. 1.

^b Strains CE1321 and CE1323 produce PhoE protein and Omp F protein, respectively, as the only major outer membrane pore protein.

^c n.t. = not tested.

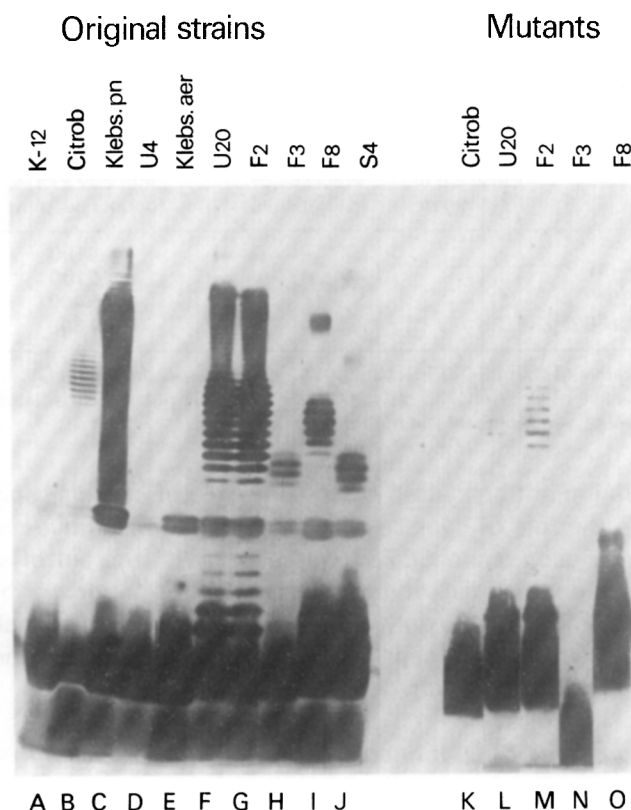


Fig. 1. LPS patterns of *E. coli* strain CE1321 (A), *Citrobacter freundii* (B, K) *Klebsiella pneumoniae* (C), *E. coli* strain U4 (D), *Klebsiella aerogenes* (E), *E. coli* strains U20 (F, L), F2 (G, M), F3 (H, N), F8 (I, O) and S4 (J). The left part of the figure (lanes A–J) shows the PhoE protein-constitutive parental strains, the right part (lanes K–O) their derivatives isolated as spontaneous mutants resistant to LPS-specific phages.

Binding of PhoE protein-specific antibodies to mutants producing rough LPS

To test whether the failure of the remaining seven strains to bind antibody is indeed caused by the presence of O-antigen, mutants with rough LPS were isolated. This was done by isolating mutants resistant to LPS-specific phages, as described in Materials and methods. As shown in Fig. 1, the mutants derived from *Citrobacter freundii* and *E. coli* strains F3 and F8 lack the O-antigen entirely whereas derivatives of *E. coli* strains U20 and F2 produce a strongly reduced amount of O-antigen. The F3 mutant apparently produces LPS with a defective core structure, since its LPS shows a reduced electrophoretic mobility (Fig. 1, lane N). These LPS mutants have acquired at the same time the ability to bind PhoE protein-specific monoclonal antibody (Table 1). These results clearly show that the presence of O-antigen-carrying LPS prevents the binding of this antibody to PhoE protein. Similar results were obtained with five other monoclonal antibodies specific for PhoE protein (results not shown) and with PhoE protein-specific polyclonal antibodies (Table 1), showing that the shielding effect of the O-antigen is quite general for surface-exposed epitopes.

Influence of the presence of capsular polysaccharide on the binding of PhoE protein-specific antibodies to whole cells

As shown in Table 1, the *Klebsiella aerogenes* strain tested, which completely lacks an O-antigen (Fig. 1), gives a significantly lower level of antibody binding than other strains with rough LPS. This strain forms large, mucoid colonies, suggesting that this

Table 2 Influence of K1 capsule and O-antigen on binding of monoclonal antibody PPI-4 to a PhoE-protein-constitutive derivative of strain SC735

Strain	Presence of O-antigen	Presence of K1 capsule	Binding of monoclonal antibody PPI-4 (counts per min)
SC735	+	+	4100
SC735 C1 ^{Ra}	+	—	2100
SC735 B3 ^{Rb}	—	+	65 400
SC735 C1 ^R B3 ^R	—	—	167 600

^a Derivative of strain SC735 resistant to K1 capsule-specific phage C1.

^b Derivative of strain SC735 resistant to LPS-specific phage B3 (isolated from local sewage, see Materials and methods).

low level of binding might be caused by the presence of capsular polysaccharide. To further investigate the role of capsule a PhoE protein constitutive mutant of the K1 capsule-producing *E. coli* strain SC735 was used. With the aid of specific phages, mutants were isolated which lack the K1 capsule, the O-antigen or both. Binding of monoclonal antibody PPI-4 was only observed for mutants which lack the O-antigen (Table 2). The presence of K1 capsular polysaccharide reduces the binding considerably, but the effect of the O-antigen is much more pronounced.

Influence of the presence of O-antigen-carrying LPS on the killing of PhoE protein-carrying cells by monoclonal antibody and complement

The results described so far clearly show that the O-antigen moiety prevents binding of antibodies to PhoE protein on whole cells as measured with the CIRA technique. However, smooth strains always synthesize some short LPS,¹ and it is therefore conceivable that PhoE protein is accessible to antibody at a few small regions per cell. This could then result in a level of antibody binding not detectable by the CIRA technique, but sufficient to cause for example complement-mediated killing of the cells. Therefore, the specific killing of PhoE protein-carrying cells by the combined action of monoclonal antibodies and complement⁵ was tested for four of the isolated PhoE protein-constitutive *E. coli* strains and their LPS mutants. No significant reduction in the number of viable cells was found for the smooth *E. coli* strains U20, F2, F3 and SC735 (Table 3). In the case of the LPS mutants, a very strong killing was found for the

Table 3 Complement-mediated killing of *E. coli* strains by treatment with PhoE protein-specific monoclonal antibody PPI-4^a

Strain	Type of LPS			
	Short		Long	
	— PPI-4	+ PPI-4	— PPI-4	+ PPI-4
U20	2	7	1	1
F2	2	3	1	1
F3	2	107 000	1	1
SC735 C1 ^R	1	10	1	1

^a Complement-mediated killing is expressed as the ratio of the number of surviving cells after treatment with heat-inactivated serum over that after treatment with complement-containing serum.

derivative of strain F3, whereas a moderate killing was found for the derivatives of strains U20 and SC735 (Table 3). The fact that smooth cells of the strains that are sensitive in the rough form are completely resistant to PhoE protein-specific monoclonals and complement strongly suggests that the shielding effect of the O-antigenic chains on PhoE protein is essentially complete.

Discussion and conclusions

The experiments described show that PhoE pore protein of the outer membrane is not accessible for antibody on whole cells of any of the enterobacterial strains tested, as long as these cells carry a normal amount of O-antigen. The shielding effect was observed for strains with several different O-serotypes and for several PhoE-protein specific monoclonal and polyclonal antibodies, showing that it is not limited to one particular type of LPS or epitope. Given the high degree of homology between the *E. coli* K-12 major porins OmpF, OmpC and PhoE^{3,4} and their immunological relation to the pore proteins of other *Enterobacteriaceae*,¹² it can be expected that the shielding effect will be found for any enterobacterial pore protein. Our results therefore strongly suggest that the prospect of using these proteins as vaccine components is not promising, since most clinical isolates of *Enterobacteriaceae* synthesize smooth LPS.¹³

The accessibility of PhoE protein to antibodies in capsule-producing strains was also investigated. Removal of the capsule by mutation resulted in only a slight increase in antibody binding, showing that the shielding effect of capsular polysaccharide is much less pronounced than the effect of O-antigen.

The results obtained with the CIRA experiments were extended by experiments in which antibody binding was tested by complement-mediated killing of PhoE protein-carrying cells. No killing was found for any of the smooth strains tested, showing that in smooth cells there are no regions of the cell surface which allow binding of sufficient antibody molecules to activate complement. Among the rough strains tested, a large variation in the sensitivity to killing by PhoE protein-specific antibody and complement was found. This might be related to the presence or absence of serum resistance factors in these strains, for example the TraT protein which was recently shown to be present in approximately 50% of clinical isolates of *E. coli*.¹⁴

Finally, it must be emphasized that our conclusions only apply to members of the *Enterobacteriaceae* and not necessarily to all Gram-negative bacteria. Indeed, it has been shown that outer membrane protein F of *Pseudomonas aeruginosa* is accessible to monoclonal antibodies in intact O-antigen-carrying cells,¹⁵ and that this protein can be used as a protective antigen in mice.⁹

Materials and methods

Bacterial strains, phages and growth conditions

The *Escherichia coli* K-12 strains CE1321 and CE1323 produce PhoE protein and OmpF protein, respectively, as the only major outer membrane pore protein.⁵ The *E. coli* strains U4 (0134), U20 (015), F2 (015), F3 (041), F8 (0156) and S4 (011) as well as *Klebsiella pneumoniae* S45, *Klebsiella aerogenes* U12 and *Citrobacter freundii* G38, have been described previously.^{12,16} The F strains have been isolated from faeces of healthy volunteers, the S strains from blood cultures of patients with *E. coli* bacteraemia, the U strains from patients with urinary tract infection. *E. coli* strain SC735 (02 K1) was obtained from P. Guinée, National Institute for Public Health, Bilthoven, The Netherlands.

Mutants that produce PhoE protein constitutively were isolated by making use of the fact that PhoE protein is co-regulated with the periplasmic enzyme alkaline phosphatase.¹⁷ After ethyl methane sulphonate mutagenesis, alkaline phosphatase-constitutive mutants were elected as green colonies on indicator plates containing 40 µg/ml of 5-bromo-4-chloro-3-indolyl-phosphate. Constitutive synthesis of PhoE protein by these mutants was checked by SDS polyacrylamide gel electrophoresis of cell envelopes and by enzyme-linked immunosorbent assay of SDS-extracted cell envelopes with PhoE protein-specific antibodies.⁵

Rough mutants were isolated as follows. For each of the smooth strains several phages were isolated from local sewage. For each strain at least one apparently LPS-specific phage was obtained, since spontaneous phage-resistant mutants of the smooth strains were found to be rough as tested by SDS polyacrylamide gel electrophoresis. The *E. coli* strain U4 turned out to be rough from the start despite having been described as O134: apparently we have used a rough derivative of this strain since the serotyping has not been repeated at the time of LPS analysis by SDS polyacrylamide gel electrophoresis.

Mutants of strain SC735 lacking the K1 capsule were isolated with the aid of K1 capsule-specific phage C1¹⁸ (obtained from P. Guinée). Absence of K1 capsule was verified by an agglutination test in K1-specific horse antiserum (obtained from P. Guinée).

All bacterial strains were grown in L. broth¹⁹ at 37°C under aeration.

Analysis of cell envelope proteins and LPS

Cell envelopes were isolated by differential centrifugation after ultrasonic disintegration of the cells.²⁰ Production of PhoE protein by the alkaline phosphatase-constitutive mutants was checked by SDS polyacrylamide gel electrophoresis.²⁰ For analysis of LPS, cell envelopes were incubated in sample buffer for 60 minutes at 60°C with 50 µg/ml of proteinase K prior to electrophoresis. The LPS was visualized in the gel by the silver stain method described by Tsai & Frasch.²¹

Binding of antibodies to intact cells

PhoE protein-specific monoclonal and polyclonal antibodies have been described previously.⁵ Binding of antibodies to intact cells was measured using the CIRA technique. Briefly, a suspension of whole cells was incubated with antibodies and, after washing, with ¹²⁵I-labeled protein A. The amount of radioactivity bound to the cells after washing was used as a measure for the amount of antibody bound to the cells.

Complement-mediated killing of cells treated with monoclonal antibody

Specific killing of PhoE protein-carrying cells by the combined action of monoclonal antibody and complement was tested as described.⁵ Normal rat serum, preadsorbed twice for 60 minutes at 4°C with approximately 10¹⁰ cells of the strain to be tested in order to prevent aspecific killing, was used as source of complement. Exponentially growing cells at a concentration of 1.5 × 10⁸ cells per ml were incubated in Veronal-buffered saline supplemented with monoclonal antibody and 10% preadsorbed rat serum. After incubation at 37°C for 30 minutes, additional rat serum was added to a final concentration of 20%. After another 30 minutes, appropriate dilutions were plated to determine the number of surviving cells. As a control, serum was used in which complement had been inactivated by incubation at 56°C for 30 minutes. Under these conditions a PhoE-constitutive *E. coli* K-12 strain gives a reduction in the number of viable cells of approximately 100 000.

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